

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
16 August 2001 (16.08.2001)

PCT

(10) International Publication Number
WO 01/59084 A1

- (51) International Patent Classification⁷: C12N 9/64, 15/85, A61K 38/48
- (21) International Application Number: PCT/US01/01221
- (22) International Filing Date: 2 February 2001 (02.02.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/181,948 11 February 2000 (11.02.2000) US
60/189,199 14 March 2000 (14.03.2000) US
- (71) Applicant (*for all designated States except US*): ELI LILLY AND COMPANY [US/US]; Lilly Corporate Center, Indianapolis, IN 46285 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): GERLITZ, Bruce, Edward [US/US]; 5551 Vicksburg Drive, Indianapolis, IN 46254 (US). GRINNELL, Brian, William [US/US]; 3625 East 71st Street, Indianapolis, IN 46220 (US). JONES, Bryan, Edward [US/US]; 14772 Beacon Park Drive, Carmel, IN 46032 (US).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:
— with international search report
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: PROTEIN C DERIVATIVES

(57) Abstract: Novel human protein C derivatives are described. These derivatives have increased anti-coagulation activity, resistance to serpin inactivation, and increased sensitivity to thrombin activation compared to wild-type protein C and retain the biological activity of the wild-type human protein C. These derivatives will require either less frequent administration and/or smaller dosage than wild-type human protein C in the treatment of acute coronary syndromes, vascular occlusive disorders, hypercoagulable states, thrombotic disorders and disease states predisposing to thrombosis.

BEST AVAILABLE COPY

-1-

Title
Protein C Derivatives

This application claims priority of Provisional Applications Serial No. 60/181,948 filed February 11, 2000 and Serial No. 60/189,199 filed March 14, 2000.

This invention relates to novel polynucleotides, polypeptides encoded by them and to the use of such polynucleotides and polypeptides. More specifically, the invention relates to human protein C derivatives with increased anti-coagulant activity, resistance to serpin inactivation, increased sensitivity to thrombin activation, or a combination thereof, when compared to wild-type activated protein C; to their production, and to pharmaceutical compositions comprising these human protein C derivatives.

Protein C is a serine protease and naturally occurring anti-coagulant that plays a role in the regulation of hemostasis by inactivating Factors V_a and VIII_a in the coagulation cascade. Human protein C is made in vivo as a single polypeptide of 461 amino acids. This polypeptide undergoes multiple post-translational modifications including, 1) cleavage of a 42 amino acid signal sequence; 2) cleavage of lysine and arginine residues (positions 156 and 157) to make a 2-chain inactive precursor or zymogen (a 155 amino acid residue light chain attached via a disulfide bridge to a 262 amino acid residue heavy chain); 3) vitamin K-dependent carboxylation of nine glutamic acid residues located within the amino-terminal 45 residues (gla-domain); and, 4) carbohydrate attachment at four sites (one in the light chain and three in the heavy chain). Finally, the 2-chain zymogen may be activated by removal of a dodecapeptide

-2-

at the N-terminus of the heavy chain, producing activated protein C (aPC) possessing greater enzymatic activity than the 2-chain zymogen.

Blood coagulation is a highly complex process regulated by the balance between pro-coagulant and anti-coagulant mechanisms. This balance determines a condition of either normal hemostasis or abnormal pathological thrombus generation and the progression, for example, of coronary thrombosis leading to acute coronary syndromes (ACS; e.g. unstable angina, myocardial infarction). Two major factors control this balance; the generation of fibrin and the activation and subsequent aggregation of platelets. Both processes are controlled by the generation of the enzyme thrombin, which occurs following activation of the clotting cascade. Thrombin, in complex with thrombomodulin, also functions as a potent anti-coagulant since it activates protein C zymogen to aPC, which in turn inhibits the generation of thrombin. Thus, through the feedback regulation of thrombin generation via the inactivation of Factors Va and VIIIa, aPC functions as perhaps the most important down-regulator of blood coagulation resulting in protection against thrombosis. In addition, aPC has anti-inflammatory properties, and exerts profibrinolytic effects that facilitate clot lysis.

Various methods of obtaining protein C from plasma and producing protein C, aPC and protein C/aPC polypeptides through recombinant DNA technology are known in the art and have been described. See e.g., U.S. Patent Nos. 4,775,624 and 5,358,932. Despite improvements in methods to produce aPC through recombinant DNA technology, aPC and derivatives thereof are difficult and costly to produce.

-3-

Unlike the zymogen protein C, activated protein C has an extremely short half-life. A major reason for the short half-life is that blood levels of aPC are regulated by molecules known as serpins (Serine Protease Inhibitors), which covalently bind to aPC forming an inactive serpin/aPC complex. The serpin/aPC complexes are formed when aPC binds and proteolytically cleaves a reactive site loop within the serpin; upon cleavage, the serpin undergoes a conformational change irreversibly inactivating aPC. The serpin/aPC complex is then eliminated from the bloodstream via hepatic receptors for the serpin/aPC complex. As a result, aPC has a relatively short half-life compared to the zymogen; approximately 20 minutes for aPC versus approximately 10 hours for human protein C zymogen (Okajima, et al., Thromb Haemost 63(1):48-53, 1990).

Therefore, an aPC derivative exhibiting resistance to serpin inactivation, while maintaining the desirable biological activities of aPC (e.g., anticoagulant, fibrinolytic, and anti-inflammatory activities), provides a compound that has an increased plasma half-life and is effectively more potent than the parent compound, requiring substantially reduced dosage levels for therapeutic applications. The potency advantages are especially important in disease states in which serpin levels are elevated.

Additionally, an aPC derivative exhibiting increased anti-coagulant activity, while maintaining the other biological activities of aPC (e.g., fibrinolytic, and anti-inflammatory activities), provides a compound that is effectively more potent than the parent compound, requiring substantially reduced dosage levels for therapeutic applications.

-4-

Enhancement of human protein C calcium and membrane binding activity by site-directed mutagenesis of the *gla*-domain has been reported by several investigators, for example, Shen et al. (J Biol. Chem., 273(47) 31086-91, 1998) and Shen et al. (Biochemistry, 36(51) 16025-31, 1997). Through continued scientific experiments, analysis, and innovation, the present inventors identified specific sites and modified targeted amino acid residues in the *gla*-domain of the aPC molecule. Surprisingly, we found increased anti-coagulant activity of the aPC derivative when specific amino acid substitutions were performed. Therefore, an aPC derivative exhibiting increased anti-coagulant activity, while maintaining the other biological activities of aPC (e.g., fibrinolytic, and anti-inflammatory activities), provides a compound that is effectively more potent than the parent compound, requiring substantially reduced dosage levels for therapeutic applications.

Furthermore, human protein C derivatives with increased sensitivity to thrombin activation (hyper-activatable zymogens) are useful as site-activated anti-thrombotic agents, as described, for example, in U.S. Patent No. 5,453,373, herein incorporated by reference, and in Richardson et al. (Protein Science, 3:711-712, 1994). Such hyper-activatable zymogens can also be constructed to contain the *gla*-domain mutants and the serpin resistant derivatives described above. These derivatives have increased anti-coagulant activity, resistance to serpin inactivation, and increased sensitivity to thrombin activation when compared to wild-type human protein C.

Accordingly, the present invention describes novel human protein C derivatives. These human protein C derivatives retain the important biological activity when

-5-

compared to wild-type protein C and have increased anti-coagulant activity, resistance to serpin inactivation, and increased sensitivity to thrombin activation when compared to wild-type human protein C. Other protein C derivatives of the present invention have increased sensitivity to thrombin activation and increased anti-coagulant activity or increased sensitivity to thrombin activation and resistance to serpin inactivation.

Therefore, these compounds provide various advantages, for example, site-activation, less frequent administration and/or smaller dosages and thus a reduction in the overall cost of production of the therapy. Thus, these compounds exhibit an advantage over current therapy in disease states of acute coronary syndromes such as unstable angina or myocardial infarction.

The present invention provides a human protein C derivative comprising SEQ ID NO: 1 wherein Asp at position 167 is substituted with Phe; Asp at position 172 is substituted with Lys and further comprising at least one amino acid substitution selected from the group consisting of:

His at position 10, Ser at position 11, or Ser at position 12 are independently substituted with any amino acid; Gln at position 32 is substituted with Glu; Asn at position 33 is substituted with Asp or Phe; and, amino acids at positions 194, 195, 228, 249, 254, 302, or 316 are substituted with an amino acid selected from Ser, Ala, Thr, His, Leu, Lys, Arg, Asn, Asp, Glu, Gly, and Gln.

The present invention also provides recombinant DNA molecules encoding the human protein C derivatives of the present invention, in particular those comprising SEQ ID NOS: 9, 10, 11, and 12.

-6-

Another aspect of the present invention provides protein sequences of these same human protein C derivatives, particularly those comprising SEQ ID NOS: 3, 4, 5, and 6, and the activated forms thereof.

The present invention comprises methods of treating acute coronary syndromes such as myocardial infarction and unstable angina.

The present invention further comprises methods of treating thrombotic disorders. Such disorders include, but are not limited to, stroke, abrupt closure following angioplasty or stent placement, and thrombosis as a result of peripheral vascular surgery.

The present invention comprises methods of treating vascular occlusive disorders and hypercoagulable states including: sepsis, disseminated intravascular coagulation, purpura fulminans, major trauma, major surgery, burns, adult respiratory distress syndrome, transplantations, deep vein thrombosis, heparin-induced thrombocytopenia, sickle cell disease, thalassemia, viral hemorrhagic fever, thrombotic thrombocytopenic purpura, and hemolytic uremic syndrome.

Another aspect of the invention comprises treating the diseases and conditions caused by or resulting from protein C deficiency as defined herein.

Another embodiment of the present invention is a method of treating sepsis comprising the administration to a patient in need thereof, a pharmaceutically effective amount of a human protein C derivative of this invention in combination with bacterial permeability increasing protein.

Another embodiment of the present invention is a method of treating thrombotic disorders which comprises: administering to a patient in need thereof a pharmaceutically effective amount of a human protein C

-7-

derivative of this invention in combination with an anti-platelet agent.

The present invention further provides a method of treating acute arterial thrombotic occlusion, thromboembolism, or stenosis in coronary, cerebral or peripheral arteries or in vascular grafts which comprises administering to a patient in need thereof a pharmaceutically effective amount of a human activated protein C in combination with a thrombolytic agent.

The present invention further provides a method of treating human patients with genetically predisposed prothrombotic disorders, for example, protein C deficiency, Factor V Leiden mutation, and prothrombin gene G20210A mutation, which comprises administering gene therapy to said patients with a recombinant DNA molecule encoding a protein C derivative.

The present invention also provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and a human protein C derivative of this invention.

Methods and aspects of producing the novel isolated human protein C derivatives are also an aspect of this invention.

The present invention also provides for the use of the human activated protein C derivatives of this invention for the manufacture of a medicament for the treatment of the above-mentioned indications

Methods and aspects of producing the novel human protein derivatives are also an aspect of this invention.

For purposes of the present invention, as disclosed and claimed herein, the following terms are as defined below.

-8-

Anti-platelet agent - one or more agents alone or in combination which reduces the ability of platelets to aggregate. Agents understood and appreciated in the art include those cited in, for example, Remington, The Science and Practice of Pharmacy, Nineteenth Edition, Vol II, pages 924-25, Mack Publishing Co., herein incorporated by reference. Such agents include but are not limited to aspirin (ASA), clopidogrel, ReoPro® (abciximab), dipyridamole, ticlopidine and IIb/IIIa antagonists.

Zymogen - protein C zymogen, as used herein, refers to secreted, inactive forms, whether one chain or two chains of protein C or derivatives thereof. Cleavage of lysine and arginine residues (positions 156 and 157) results in a 2-chain (heavy and light) inactive zymogen.

Activated protein C refers to the activated form of protein C zymogen which is produced after by removal of a dodecapeptide at the N-terminus of the heavy chain, producing activated protein C.

Activated protein C or aPC refers to recombinant aPC. aPC includes and is preferably recombinant human aPC although aPC may also include other species having protein C proteolytic, amidolytic, esterolytic, and biological (anti-coagulant, anti-inflammatory, or pro-fibrinolytic) activities.

Human protein C derivative(s) refers to the recombinantly produced derivatives of this invention that differ from wild-type human protein C but when activated retain the essential properties i.e., proteolytic, amidolytic, esterolytic, and biological (anti-coagulant, anti-inflammatory, pro-fibrinolytic activities). The definition of human protein C derivatives as used herein

-9-

also includes the activated form of the above-identified human protein C derivatives.

Treating - describes the management and care of a patient for the purpose of combating a disease, condition, or disorder whether to eliminate the disease, condition, or disorder, or prophylactically to prevent the onset of the symptoms or complications of the disease, condition, or disorder.

Continuous infusion - continuing substantially uninterrupted the introduction of a solution or suspension into a vein for a specified period of time.

Bolus injection - the injection of a drug in a defined quantity (called a bolus) over a period of time up to about 120 minutes.

Suitable for administration - a lyophilized formulation or solution that is appropriate to be given as a therapeutic agent.

Unit dosage form - refers to physically discrete units suitable as unitary dosages for human subjects, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient.

Hypercoagulable states - excessive coagulability associated with disseminated intravascular coagulation, pre-thrombotic conditions, activation of coagulation, or congenital or acquired deficiency of clotting factors such as aPC.

Protein C deficiency - protein C deficiency as used herein can be congenital or acquired. For either type, the protein C level in circulation is below the lower limit of the normal range. Skilled artisans realize that the normal range is established by a standard protocol utilizing FDA

-10-

approved equipment and diagnostic kits for determining protein C levels.

Pharmaceutically effective amount - a therapeutically efficacious amount of a pharmaceutical compound. The particular dose of the compound administered according to this invention will, of course, be determined by the attending physician evaluating the particular circumstances surrounding the case, including the compound administered, the particular condition being treated, the patient characteristics and similar considerations.

Acute coronary syndromes - clinical manifestations of coronary atherosclerosis complicated by coronary plaque rupture, superimposed coronary thrombosis, and jeopardized coronary blood flow resulting in coronary ischemia and/or myocardial infarction. The spectrum of acute coronary syndromes includes unstable angina, non-Q-wave (i.e., non-ST-segment elevation) myocardial infarction, and Q-wave (i.e., ST-segment elevation) myocardial infarction.

Gene Therapy - A therapeutic regime which includes the administration of a vector containing DNA encoding a therapeutic protein, directly to affected cells where the therapeutic protein will be produced. Target tissue for gene delivery include, for example, skeletal muscle, vascular smooth muscle, and liver. Vectors include, for example, plasmid DNA, liposomes, protein-DNA conjugates, and vectors based on adenovirus or herpes virus. Gene therapy has been described, for example, by Kessler et al., PNAS, USA, 93:14082-87, 1996.

Thrombotic disorders - a disorder relating to, or affected with the formation or presence of a blood clot within a blood vessel. Such disorders include, but are not limited to, stroke, abrupt closure following angioplasty or

-11-

stent placement, and thrombosis as a result of peripheral vascular surgery.

Purpura fulminans - ecchymotic skin lesions, fever, hypotension associated with bacterial sepsis, viral, bacterial or protozoan infections. Disseminated intravascular coagulation is usually present.

Tissue factor pathway inhibitor (TFPI) . . refers to naturally or recombinant forms of TFPI. This protein is believed to block tissue-mediated clotting in small blood vessels, which potentially leads to organ failure and death.

Serpin - any of a group of structurally related proteins that typically are serine protease inhibitors whose inhibiting activity is conferred by a reactive site in a highly variable and mobile peptide loop and that include but are not limited to protein C inhibitor (PCI) and α_1 -antitrypsin (α_1 -AT).

Inhibitor recognition sequence S2: the 2nd residue N-terminal to the cleavage site of PCI or α_1 -AT.

Inhibitor recognition sequence S3': the 3rd residue C-terminal to the cleavage site of PCI or α_1 -AT.

Inhibitor recognition sequence S4': the 4th residue C-terminal to the cleavage site of PCI or α_1 -AT.

Wild-type protein C - the type of protein C that predominates in a natural population of humans in contrast to that of natural or laboratory mutant polypeptide forms of protein C.

Bactericidal permeability increasing protein - includes naturally and recombinantly produced bactericidal permeability increasing (BPI) protein; natural, synthetic, and recombinant biologically active polypeptide fragments of BPI protein; biologically active polypeptide variants of BPI protein or fragments thereof, including hybrid fusion proteins and dimers; biologically active variant analogs of BPI protein or fragments or variants thereof, including

-12-

cysteine-substituted analogs; and BPI-derived peptides. The complete amino acid sequence of human BPI, as well as the nucleotide sequence of DNA encoding BPI have been elucidated by Gray, et al., 1989, J. Biol. Chem 264:9505. Recombinant genes encoding and methods for expression of BPI proteins, including BPI holoprotein and fragments of BPI are disclosed in U.S. Patent No. 5,198,541, herein incorporated by reference.

The phrase "in combination with" as used herein, refers to the administration of additional agents with human aPC derivatives either simultaneously, sequentially or a combination thereof. Examples of additional agents are anti-platelet agents, thrombolytic agents, and BPI protein.

The amino acid abbreviations are accepted by the United States Patent and Trademark Office as set forth in 37 C.F.R. 1.822 (d)(1) (1998).

The present invention provides human protein C derivatives, which have increased anti-coagulant activity, resistance to serpin inactivation, and increased sensitivity to thrombin activation as compared to wild-type protein C and the use of these derivatives in the zymogen form as well as in the activated form. The activated form of human protein C derivatives may be produced by activating recombinant human protein C derivative zymogen *in vitro* or by direct secretion of the activated form of protein C. The means by which the activation occurs is not critical and the process aspects of this invention include any and all means of activation. Human protein C derivatives may be produced in eukaryotic cells, transgenic animals, or transgenic plants, including, for example, secretion from human kidney 293 cells or AV 12 cells as a zymogen, then purified and activated by techniques known to the skilled artisan.

-13-

Preferred human protein C derivatives of the present invention include S11G:Q32E:N33D:D167F:D172K:L194S, S11G:Q32E:N33D:D167F:D172K:L194S:T254S, S11G:Q32E:N33D:D167F:D172K, and H10Q:S11G:Q32E:N33D:D167F:D172K.

Human protein C derivative

S11G:Q32E:N33D:D167F:D172K:L194S contains a glycine residue at position 11 instead of the serine residue normally found at this position, a glutamic acid residue at position 32 instead of the glutamine residue normally found at this position an aspartic acid residue at position 33 instead of the asparagine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, a lysine at position 172 rather than the aspartic acid normally found at this position and a serine residue at position 194 instead of the leucine residue normally found at this position. Other preferred amino acid substitutions for positions 194 include Ser, Ala, Thr, His, Leu, Lys, Arg, Asn, Asp, Glu, Gly, and Gln and any amino acid for position 11.

Human protein C derivative

S11G:Q32E:N33D:D167F:D172K:L194S:T254S contains a glycine residue at position 11 instead of the serine residue normally found at this position, a glutamic acid residue at position 32 instead of the glutamine residue normally found at this position an aspartic acid residue at position 33 instead of the asparagine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, a lysine at position 172 rather than the aspartic acid normally found at this position, a serine residue at position 194 instead of the leucine residue normally found at this position, and a

-14-

serine residue at position 254 instead of the threonine residue normally found at this position. Other preferred amino acid substitutions for positions 194 and 254 include Ser, Ala, Thr, His, Leu, Lys, Arg, Asn, Asp, Glu, Gly, and Gln and any amino acid for position 11.

Human protein C derivative S11G:Q32E:N33D:D167F:D172K contains a glycine residue at position 11 instead of the serine residue normally found at this position, a glutamic acid residue at position 32 instead of the glutamine residue normally found at this position an aspartic acid residue at position 33 instead of the asparagine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, and a lysine at position 172 rather than the aspartic acid normally found at this position. Other preferred amino acid substitutions for positions 11 include any amino acid.

Human protein C derivative H10Q:S11G:Q32E:N33D:D167F:D172K preferably contains a glutamine at position 10 rather than the histidine residue normally found at this position, a glycine at position 11 rather than the serine normally found at this position, a glutamic acid residue at position 32 instead of the glutamine residue normally found at this position an aspartic acid residue at position 33 instead of the asparagine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, and a lysine at position 172 rather than the aspartic acid normally found at this position. Other preferred amino acid substitutions for positions 11 include any amino acid.

Other embodiments of the present inventions include H10Q:S11G:S12K:D167F:D172K:L194S:T254S,

-15-

S11G:Q32E:D167F:D172K:L194S,
S11G:Q32E:D167F:D172K:L194S:T254S,
S11G:Q32E:N33F:D167F:D172K:L194S, and
S11G:Q32E:N33F:D167F:D172K:L194S:T254S, and activated forms thereof which have increased anti-coagulation activity and resistance to serpin inactivation, and increased sensitivity to thrombin activation, as compared to wild-type activated protein C.

Human protein C derivative

H10Q:S11G:S12K:D167F:D172K:L194S:T254S preferably contains a glutamine at position 10 rather than the histidine residue normally found at this position, a glycine at position 11 rather than the serine normally found at this position, a lysine residue at position 12 rather than a serine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, a lysine at position 172 rather than the aspartic acid normally found at this position, a serine at position 194 rather than the leucine normally found at this position, and a serine at position 254 instead of a threonine normally found at this position. Other preferred amino acid substitutions for positions Other preferred amino acid substitutions for positions 194 and 254 include Ser, Ala, Thr, His, Leu, Lys, Arg, Asn, Asp, Glu, Gly, and Gln and any amino acid for positions 10, 11, and 12.

Human protein C derivative S11G:Q32E:D167F:D172K:L194S contains a glycine residue at position 11 instead of the serine residue normally found at this position, a glutamic acid residue at position 32 instead of the glutamine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, a lysine at position 172 rather than the aspartic

-16-

acid normally found at this position, and a serine residue at position 194 instead of the leucine residue normally found at this position. Other preferred amino acid substitutions for positions 194 include Ser, Ala, Thr, His, Leu, Lys, Arg, Asn, Asp, Glu, Gly, and Gln and any amino acid for position 11.

Human protein C derivative

S11G:Q32E:D167F:D172K:L194S:T254S contains a glycine residue at position 11 instead of the serine residue normally found at this position, a glutamic acid residue at position 32 instead of the glutamine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, a lysine at position 172 rather than the aspartic acid normally found at this position, a serine residue at position 194 instead of the leucine residue normally found at this position, and a serine residue at position 254 instead of the threonine residue normally found at this position. Other preferred amino acid substitutions for positions 194 and 254 include Ser, Ala, Thr, His, Leu, Lys, Arg, Asn, Asp, Glu, Gly, and Gln and any amino acid for position 11.

Human protein C derivative

S11G:Q32E:N33F:D167F:D172K:L194S contains a glycine residue at position 11 instead of the serine residue normally found at this position, a glutamic acid residue at position 32 instead of the glutamine residue normally found at this position a phenylalanine residue at position 33 instead of the asparagine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, a lysine at position 172 rather than the aspartic acid normally found at this position, and a serine residue at position 194 instead of

-17-

the leucine residue normally found at this position. Other preferred amino acid substitutions for positions 194 include Ser, Ala, Thr, His, Leu, Lys, Arg, Asn, Asp, Glu, Gly, and Gln and any amino acid for position 11.

Human protein C derivative

S11G:Q32E:N33F:D167F:D172K:L194S:T254S contains a glycine residue at position 11 instead of the serine residue normally found at this position, a glutamic acid residue at position 32 instead of the glutamine residue normally found at this position a phenylalanine residue at position 33 instead of the asparagine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, a lysine at position 172 rather than the aspartic acid normally found at this position, a serine residue at position 194 instead of the leucine residue normally found at this position, and a serine residue at position 254 instead of the threonine residue normally found at this position. Other preferred amino acid substitutions for positions 194 and 254 include Ser, Ala, Thr, His, Leu, Lys, Arg, Asn, Asp, Glu, Gly, and Gln and any amino acid for position 11.

Further embodiments of the present invention include human protein C derivatives: S11G:D167F:D172K:L194S, S11G:D167F:D172K:L194S:T254S, S11G:S12K:D167F:D172K:L194S, S12K:D167F:D172K, D167F:D172K:L194S:T254S, S12K:D167F:D172K:L194S, S12K:D167F:D172K:L194S:T254S, Q32E:N33D:D167F:D172K, S11G:Q32E:D167F:D172K, S11G:Q32E:N33F:D167F:D172K, and activated forms thereof which have increased anti-coagulant activity, resistance to inactivation by serpins, increased sensitivity to thrombin activation or combinations of these activities as compared to wild-type human activated protein C.

-18-

Human protein C derivative S11G:D167F:D172K:L194S preferably contains a glycine residue at position 11 rather than a serine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, a lysine at position 172 rather than the aspartic acid normally found at this position, and a serine at position 194 rather than the leucine normally found at this position. Other preferred amino acid substitutions for positions 194 include Ser, Ala, Thr, His, Leu, Lys, Arg, Asn, Asp, Glu, Gly, and Gln and any amino acid for position 11.

Human protein C derivative S11G:D167F:D172K:L194S:T254S preferably contains a glycine residue at position 11 rather than a serine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, a lysine at position 172 rather than the aspartic acid normally found at this position, a serine at position 194 rather than the leucine normally found at this position, and a serine at position 254 instead of a threonine normally found at this position. Other preferred amino acid substitutions for positions 194 and 254 include Ser, Ala, Thr, His, Leu, Lys, Arg, Asn, Asp, Glu, Gly, and Gln and any amino acid for position 11.

Human protein C derivative S11G:S12K:D167F:D172K:L194S preferably contains a glycine residue at position 11 rather than a serine residue normally found at this position, a lysine residue at position 12 rather than a serine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, a lysine at position 172 rather than the aspartic acid normally found at this position, and a serine at position 194 rather than the leucine normally found at this

-19-

position. Other preferred amino acid substitutions for positions 194 include Ser, Ala, Thr, His, Leu, Lys, Arg, Asn, Asp, Glu, Gly, and Gln and any amino acid for position 11 and 12.

Human protein C derivative S12K:D167F:D172K preferably contains a lysine residue at position 12 rather than a serine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, and a lysine at position 172 rather than the aspartic acid normally found at this position. Other preferred amino acid substitutions for positions 12 include any amino acid.

Human protein C derivative D167F:D172K:L194S:T254S preferably contains a phenylalanine at position 167 rather than the aspartic acid normally found at this position, a lysine at position 172 rather than the aspartic acid normally found at this position, a serine at position 194 rather than the leucine normally found at this position, and a serine at position 254 instead of a threonine normally found at this position. Other preferred amino acid substitutions for positions 194, and 254 include Ser, Ala, Thr, His, Leu, Lys, Arg, Asn, Asp, Glu, Gly, and Gln.

Human protein C derivative S12K:D167F:D172K:L194S preferably contains a lysine residue at position 12 rather than a serine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, a lysine at position 172 rather than the aspartic acid normally found at this position, and a serine at position 194 rather than the leucine normally found at this position. Other preferred amino acid substitutions for positions 194 include Ser, Ala,

-20-

Thr, His, Leu, Lys, Arg, Asn, Asp, Glu, Gly, and Gln and any amino acid for position 11.

Human protein C derivative S12K:D167F:D172K:L194S:T254S preferably contains a lysine residue at position 12 rather than a serine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, a lysine at position 172 rather than the aspartic acid normally found at this position, a serine at position 194 rather than the leucine normally found at this position, and a serine at position 254 instead of a threonine normally found at this position. Other preferred amino acid substitutions for positions 194 and 254 include Ser, Ala, Thr, His, Leu, Lys, Arg, Asn, Asp, Glu, Gly, and Gln and any amino acid for position 12.

Human protein C derivative Q32E:N33D:D167F:D172K contains a glutamic acid residue at position 32 instead of the glutamine residue normally found at this position an aspartic acid residue at position 33 instead of the asparagine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, and a lysine at position 172 rather than the aspartic acid normally found at this position.

Human protein C derivative S11G:Q32E:D167F:D172K contains a glycine residue at position 11 instead of the serine residue normally found at this position, a glutamic acid residue at position 32 instead of the glutamine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, and a lysine at position 172 rather than the aspartic acid normally found at this position. Other

-21-

preferred amino acid substitutions for positions 11 include any amino acid

Human protein C derivative S11G:Q32E:N33F:D167F:D172K contains a glycine residue at position 11 instead of the serine residue normally found at this position, a glutamic acid residue at position 32 instead of the glutamine residue normally found at this position a phenylalanine residue at position 33 instead of the asparagine residue normally found at this position, a phenylalanine at position 167 rather than the aspartic acid normally found at this position, and a lysine at position 172 rather than the aspartic acid normally found at this position. Other preferred amino acid substitutions for position 11 include any amino acid.

In addition, human protein C derivatives of the present invention include additional deletions, additions, or substitutions of amino acid residues of the protein C derivatives described above, but which result in changes that do not effect the basic characteristics of this invention. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. Thus, the derivatives of the present invention include derivatives having an amino acid sequence that vary from SEQ ID NOS: 3, 4, 5, and 6, by conservative substitutions i.e., those that substitute a residue with another of like characteristics. Typical substitutions are among Ala, Val, Leu and Ile; among Ser and Thr; among the acidic residues Asp and Glu; among Asn and Gln; and among the basic residues Lys and Arg; or aromatic residues Phe and Tyr. Other derivatives are those in which several, 5-10, 1-5, or 1-2 amino acids are substituted, deleted, or added in any combination. A preferred

-22-

embodiment is based on SEQ ID NO: 1 includes the addition of the 42 amino acid signal peptide sequence as illustrated in Figure 1 and shown in SEQ ID NO: 2.

Preferably, the human protein C derivatives of the present invention are not further substituted or modified. That is, substitutions are limited to the derivatives of the present invention.

The invention also provides DNA compounds for use in making the human protein C derivatives. These DNA compounds comprise the coding sequence for the light chain of human protein C zymogen or human protein C derivative zymogen positioned immediately adjacent to, downstream of, and in translational reading frame with the prepropeptide sequence of human protein C zymogen or human protein C derivative zymogen. The DNA sequences preferably encode the Lys-Arg dipeptide which is processed during maturation of the protein C molecule, the activation peptide and the heavy chain of the human protein C derivative. Thus, the human protein C derivatives of the present invention are variant or mutant polypeptides which contain at least 3, preferably 3 to 7 amino acids, which differ from the wild-type protein C sequence identified as SEQ ID NO: 1 (which does not contain the 42 amino acid signal sequence) or the corresponding wild-type amino acid in SEQ ID NO: 2 (which contains the 42 amino acid signal sequence). Thus, one skilled in the art recognizes that a human protein C derivative which differs from the amino acid sequence of the wild-type protein C sequence identified as SEQ ID NO: 1 inherently corresponds to the wild-type protein C sequence identified as SEQ ID NO: 2 at the amino acid position determined after removal of the 42 amino acid signal sequence. Furthermore, one skilled in the art recognizes

-23-

that prior to activation, the cleavage of the lysine and arginine residues (positions 156 and 157) occurs.

Those skilled in the art will recognize that, due to the degeneracy of the genetic code, a variety of DNA compounds can encode the derivatives described above. U.S. Patent No. 4,775,624, the entire teaching of which is herein incorporated by reference, discloses the wild-type form of the human protein C molecule. The skilled artisan could readily determine which changes in the DNA sequences which could encode the exact derivatives as disclosed herein. The invention is not limited to the specific DNA sequences disclosed. Consequently, the construction described below and in the accompanying Examples for the preferred DNA compounds are merely illustrative and do not limit the scope of the invention.

All of the DNA compounds of the present invention were prepared by the use of site-directed mutagenesis to change particular positions within the human protein C zymogen. The technique for modifying nucleotide sequences by site-directed mutagenesis is well known to those skilled in the art. See e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning : A Laboratory Manual, second Edition (1989).

The human protein C derivatives can be made by techniques well known in the art utilizing eukaryotic cell lines, transgenic animals, or transgenic plants. Skilled artisans will readily understand that appropriate host eukaryotic cell lines include but are not limited to HepG2, LLC-MK₂, CHO-K1, 293, or AV12 cells, examples of which are described in U.S. Patent No. 5,681,932, herein incorporated by reference. Furthermore, examples of transgenic production of recombinant proteins are described in U.S.

-24-

Patent Nos. 5,589,604 and 5,650,503, herein incorporated by reference.

Skilled artisans recognize that a variety of vectors are useful in the expression of a DNA sequence of interest in a eukaryotic host cell. Vectors that are suitable for expression in mammalian cells include, but are not limited to: pGT-h, pGT-d; pCDNA 3.0, pCDNA 3.1, pCDNA 3.1+Zeo, and pCDNA 3.1+Hygro (Invitrogen); and, pIRES/Hygro, and pIRES/neo (Clontech). The preferred vector of the present invention is pIG3 as described in Example 1.

Other sequences may also be desirable which allow for regulation of expression of the protein sequences relative to the growth of the host cell. Such regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

In some cases it may be necessary to modify the coding sequence so that it may be attached to the control sequences with the appropriate orientation; i.e. to maintain the proper reading frame.

The human protein C derivatives made by any of these methods must undergo post-translational modifications such as the addition of nine or ten gamma-carboxy-glutamates, the

-25-

addition of one erythro-beta-hydroxy-Asp (beta-hydroxylation), the addition of four Asn-linked oligosaccharides (glycosylation) and, the removal of the leader sequence (42 amino acid residues). Such post-translational modifications are necessary for efficient production and secretion of the protein C derivatives from mammalian cells.

It is known in the art that post-translational modifications of recombinant proteins such as the human protein C derivatives of the present invention may vary depending on which host cell line is utilized for the expression of the recombinant protein. For example, the post-translational modification of gamma-carboxylation, which is essential for the anti-coagulant activity of the human protein C derivatives of the present invention, may be higher, slightly lower, or much lower than plasma derived wild-type protein C gamma-carboxylation, depending on the host cell line used (Yan et al., Bio/Technology 8(7):655-661, 1990). Such differences in gamma-carboxylation provide a basis for the use of site-directed mutagenesis to change particular positions within the human protein C molecule that will result in an increase in anti-coagulant activity.

The human protein C derivatives of the present invention may be administered as a zymogen or in the activated form. Methods for the activation of zymogen forms of human protein C and human protein C derivatives to activated human protein C and activated human protein C derivatives are old and well known in the art. Human protein C may be activated by thrombin alone, by a thrombin/thrombomodulin complex, by RVV-X, a protease from Russell's Viper venom, by pancreatic trypsin or by other proteolytic enzymes.

-26-

The present invention further provides for the treatment of acute coronary syndromes comprising myocardial infarction, and unstable angina with human protein C derivatives with increased anti-coagulation activity, resistance to serpin inactivation, and increased sensitivity to thrombin activation as compared to wild-type aPC.

The recombinant human protein C derivatives of the present invention are also useful for the treatment of thrombotic disorders such as stroke, abrupt closure following angioplasty or stent placement, and thrombosis as a result of peripheral vascular surgery.

Additionally, the recombinant human protein C derivatives of the present invention are useful for the treatment of vascular occlusive disorders or hypercoagulable states associated with sepsis, disseminated intravascular coagulation, major trauma, major surgery, burns, adult respiratory distress syndrome, transplantations, deep vein thrombosis, heparin-induced thrombocytopenia, sickle cell disease, thalassemia, viral hemorrhagic fever, thrombotic thrombocytopenic purpura, and hemolytic uremic syndrome. In another embodiment, the recombinant human protein C derivatives of the present invention are useful for the treatment of sepsis in combination with bacterial permeability increasing protein. In yet another aspect of this invention the activated human protein C derivatives of the present invention are combined with an anti-platelet agent(s) to treat or prevent various disorders, such as, thrombotic disease.

In another embodiment, the recombinant human protein C derivatives of the present invention are useful for the treatment of sepsis in combination with tissue factor pathway inhibitor.

-27-

Another aspect of the invention comprises treating the diseases and conditions caused or resulting from protein C deficiency as defined herein. This aspect of the invention contemplates any and all modifications to any aPC molecule resulting in increased anti-coagulant activity and resistance to serpin inactivation as compared to wild-type aPC.

The recombinant human protein C derivatives of the present invention are useful for the treatment of acute arterial thrombotic occlusion, thromboembolism, or stenosis in coronary, cerebral or peripheral arteries or in vascular grafts, in combination with a thrombolytic agent such as tissue plasminogen activator, streptokinase, and related compounds or analogs thereof.

The human protein C derivatives can be formulated according to known methods to prepare a pharmaceutical composition comprising as the active agent an aPC derivative and a pharmaceutically acceptable bulking agent. For example, a desired formulation would be one that is a stable lyophilized product of high purity comprising a bulking agent such as sucrose, trehalose or raffinose; a salt such as sodium chloride or potassium chloride; a buffer such as sodium citrate, Tris acetate, or sodium phosphate, at a pH of about 5.5 to about 6.5; and an activated human protein C derivative.

The human aPC derivatives of the present invention can be administered at an appropriate dose level understood and appreciated in the art and determined by the attending physician evaluating the particular circumstances surrounding the case. Preferably, the amount of human aPC derivative administered will be from about 0.01 $\mu\text{g/kg/hr}$ to about 50 $\mu\text{g/kg/hr}$. More preferably, the amount of human aPC

-28-

derivative administered will be about 0.1 $\mu\text{g/kg/hr}$ to about 25 $\mu\text{g/kg/hr}$. Yet even more preferably the amount of human aPC derivative administered will be about 0.1 $\mu\text{g/kg/hr}$ to about 15 $\mu\text{g/kg/hr}$. Even more preferably the amount of human aPC derivative administered will be about 1 $\mu\text{g/kg/hr}$ to about 15 $\mu\text{g/kg/hr}$. The most preferable amounts of human aPC derivative administered will be about 5 $\mu\text{g/kg/hr}$ or about 10 $\mu\text{g/kg/hr}$.

Preferably, the human aPC derivatives will be administered parenterally to ensure delivery into the bloodstream in an effective form by injecting a dose of 0.01 mg/kg/day to about 1.0 mg/kg/day, one to six times a day, for one to ten days. More preferably, the human aPC derivatives will be administered B.I.D. (2 times a day) for three days.

Alternatively, the human aPC derivatives will be administered at a dose of about 0.01 $\mu\text{g/kg/hr}$ to about 50 $\mu\text{g/kg/hr}$, by continuous infusion for 1 to 240 hours.

The preferred plasma ranges obtained from the amount of human protein C derivative administered will be 0.02 ng/ml to less than 100 ng/ml.

In another alternative, the human protein C derivatives will be administered by injecting a portion (1/3 to 1/2) of the appropriate dose per hour as a bolus injection over a time from about 5 minutes to about 120 minutes, followed by continuous infusion of the appropriate dose for up to 240 hours.

In another alternative, the human protein C derivatives will be administered by local delivery through an intracoronary catheter as an adjunct to high-risk angioplasty (with and without stenting, and with or without combination therapy with anti-platelet agents). The amount

-29-

of human protein C derivative administered will be from about 0.01 mg/kg/day to about 1.0 mg/kg/day by continuous infusion, bolus injection, or a combination thereof.

In another alternative, the human protein C derivatives will be administered subcutaneously at a dose of 0.01 mg/kg/day to about 10.0 mg/kg/day, to ensure a slower release into the bloodstream. Formulation for subcutaneous preparations will be done using known methods to prepare such pharmaceutical compositions.

The human protein C derivatives described in this invention have increased anti-coagulant activity, resistance to serpin inactivation, and increased sensitivity to thrombin activation. Therefore, these compounds provide various advantages over conventional therapeutic agents, for example, site-activation, less frequent administration and/or smaller dosages, increased efficacy, and thus a reduction in the overall cost of production of the therapy.

The following Examples are provided merely to further illustrate the present invention. The scope of the invention shall not be construed as merely consisting of the following Examples.

Example 1

Protein C Derivative Construction and Production

Human protein C derivatives were constructed using the polymerase chain reaction (PCR) following standard methods. The source of the wild-type coding sequence was plasmid pLPC (Bio/Technology 5:1189-1192, 1987). The universal PCR primers used include: PC001b; 5'-GCGATGTCTAGAccaccATGTGGCAGCTCACAAGCCTCCTGC -3', which encodes for an XbaI restriction site (underlined) used for subcloning, a Kozak consensus sequence (lowercase) (Kozak, J

-30-

Cell Biol 108(2):229-41, 1989), and the 5' end of the coding region for protein C: PC002e; 5'-CAGGGATGATCACTAAGGTGCCCAGCTCTTCTGG-3', which encodes for the 3' end of the coding region for human protein C, and includes a BclI restriction site (underlined) for subcloning. All site-directed mutagenesis was accomplished by established PCR methodology, using complementary oligonucleotides containing the desired sequence changes. The first round of PCR was used to amplify two fragments of the protein C gene; the 5' fragment was generated using PC001b and the antisense mutagenic primer, and the 3' fragment was generated using PC002e and the sense mutagenic primer. The resulting amplified products were purified by standard procedures. These fragments were combined and then used as a template for a second round of PCR using primers PC001b and PC002e. The final PCR product was digested with XbaI and BclI and subcloned into similarly digested expression vector pIG3. A wild-type construct was similarly generated by PCR using the two universal primers and the plasmid pLPC as the template, followed by subcloning into pIG3. The mutations were confirmed by DNA sequencing of both the coding and non-coding strands. The pIG3 vector was generated by the insertion of an "internal ribosome entry site" (IRES) (Jackson, et al., Trends Biochem Sci 15(12):447-83, 1990) and green fluorescent protein (GFP) (Cormack, et al., Gene 173:33-38, 1996) gene into the mammalian expression vector pGTD (Gerlitz, et al., Biochem J 295(Pt 1):131-40, 1993). When a cDNA of interest is cloned into the multiple cloning site of pIG3, the GBMT promoter (Berg, et al., Nucleic Acids Res 20(20):5485-6, 1992) drives expression of a bicistronic mRNA (5'- cDNA - IRES - GFP - 3'). Efficient translation of the first cistron is

-31-

initiated by classical assembly of ribosome subunits on the 5'-methylated cap structure of the mRNA; while the normally inefficient translation of a second cistron is overcome by the IRES sequence which allows for internal ribosome assembly on the mRNA. The coupling of the cDNA and reporter on a single mRNA, translated as separate proteins, allows one to screen for the highest-producing clones on the basis of fluorescence intensity. The expression vector also contains an ampicillin resistance cassette for maintenance of the plasmid in *E. coli*, and a murine DHFR gene with appropriate expression sequences for selection and amplification purposes in mammalian tissue expression.

The adenovirus-transformed Syrian hamster AV12-664 cell line was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 µg/mL gentamicin, 200 µg /mL Geneticin (G418), and 10 µg /mL vitamin K1. One day prior to transfection, cells were plated at a density of about 10^5 cells/ 25 cm². FspI-linearized plasmids were transfected using either the calcium phosphate method (ProFection, Gibco BRL-Life Technologies) or FuGene-6 (Boehringer Mannheim), following the manufacturer's instructions. Approximately 48 hours after transfection, the medium was replaced with medium containing 250 nM methotrexate for selection. Colonies resistant to methotrexate were pooled 2-3 weeks after applying drug selection and expanded. The pools were subjected to fluorescence activated cell sorting based upon GFP fluorescence intensity (Cormack, 1996), with the most intense 5% of fluorescent cells being retained and expanded. To obtain material for purification, recombinant cells were grown in a modified mixture of Dulbecco's modified Eagle's and Ham's F-12 media (1:3) containing 1 µg/mL human insulin,

-32-

1 µg/mL human transferrin, and 10 µg/mL vitamin K1. Conditioned media were collected, adjusted to a final concentration of 5 mM benzamidine and 5 mM EDTA, pH 8.0, and protein C was purified via anion-exchange chromatography as described (Yan, et al., Bio/Technology 8:655-661, 1990). Purified protein was desalted/concentrated in Ultrafree-CL 30,000 NMWL filtration units (Millipore) using Buffer A (150 mM NaCl, 20 mM Tris-HCl, pH 7.4), and quantitated by Pierce BCA assay using bovine serum albumin (BSA) as the standard.

Example 2
Serpin Resistant Mutants

The use of site-directed mutagenesis to change particular positions within human protein C molecule that decrease inactivation by serpins, and consequently result in extended plasma half-lives is described. The recognition sequences in the two primary aPC inhibitors α_1 -AT and PCI reveal some differences that can be exploited by altering the residues in aPC that interact with these sequences. Table I depicts the sequences recognized by aPC. The cleavage site occurs between the two residues shown in italics. Residues occupying the specific subsites, S2, S3', and S4', are underlined.

In general, the recognized sites in factor Va are different from the sites in either factor VIIIa or the inhibitors, therefore, it is possible to engineer the active site of aPC to preferentially cleave the more critical coagulant factor Va, while at the same time decrease aPC's likelihood of being inhibited by serpins.

-33-

Table I.

Coagulation Factors		S2	...S3.S4.
Factor Va	300-313	NCPKKI <i>R</i>	N L K K I T R
Factor Va	500-513	SRSLDR <i>R</i>	G I Q R A A A
Factor Va	673-685	STVMAI <i>R</i>	K M H D R L E
Factor VIIIa	330-341	PEEPQL <i>R</i>	M K N N E E A
Factor VIIIa	560-571	KESVDQ <i>R</i>	G N Q I M S D
Serpins			
PCI		GTIFTE <i>R</i>	S A R L N S Q
α_1 -AT		FLEAIP <i>M</i>	S I P P E V K

In particular, three sites of recognition within the active site show distinctive differences between substrate recognition sequences and inhibitor recognition sequences: S2 (the 2nd residue N-terminal to the cleavage site), S3' site, and S4'. The S2 site is primarily occupied by polar residues in the factor Va sequences; unlike PCI and α_1 -AT, which have hydrophobic residues at this position. The S3' site occupied by polar side chains in all of the substrate sequences, but notably, a hydrophobic side chain in the α_1 -AT sequence. The S4' site is occupied by charged residues in all three factor Va sequences, but is occupied by hydrophobic residues in the factor VIIIa and inhibitor sequences.

Based upon the crystal structures of the PPACK-inhibited aPC (Mather, et al., EMBO J. 15(24):6822-6831, 1996) and Hirulog 3-inhibited thrombin (Qiu, et al., Biochemistry 31(47):11689-97, 1992), two aPC-substrate model structures were created and energy minimized using a CHARMM protocol:

-34-

(1) The sequence representing the factor Va R506 cleavage sequence.

(2) The recognition sequence of α_1 -AT, with the Met substituted with Arg (corresponding to a polypeptide of α_1 -AT which exhibits extremely high affinity for aPC).

These models allowed for the identification of residues which form critical contacts in these three specific sites. A summary of residues which may form specific contacts within the active site, and replacements that are expected to provide enhanced specificity and/or activity are summarized in Table II. In general, mutations of residues that form contacts within the specific subsites of the active site are designed to reflect changes in the environment to drive the specificity of human protein C derivatives away from the recognition of the two primary physiological inhibitors, and potentially enhance human protein C derivative's proteolytic activity.

Table II. Mutations constructed for alteration of specificity

Site	aPC Residue	Constructed replacements	Substrate Contact
S2	Thr254	Ser	Aliphatic part of sidechain
S3'	Tyr302	Glu, Gln	End of sidechain
S4'	Leu194	Ser, Thr, Ala	Aliphatic part of sidechain
S4'	Ala195	Gly	Aliphatic part of sidechain
S4'	Leu228	Gln	End of sidechain
S4'	Phe316	Asn	Aliphatic part of sidechain

-35-

Example 3Activation of Recombinant Protein C

Complete activation of the zymogen forms of protein C and derivatives was accomplished by incubation with thrombin-sepharose. Thrombin-sepharose was washed extensively with Buffer A. 200 μ L of packed thrombin-sepharose was mixed with 250 μ g of protein C in 1 mL of the same buffer and incubated at 37°C for 4 hours with gentle shaking on a rotating platform. During the course of the incubation, the degree of protein C activation was monitored by briefly pelleting the thrombin-sepharose, and assaying a small aliquot of the supernatant for aPC activity using the chromogenic substrate S-2366 (DiaPharma). Following complete activation, the thrombin-sepharose was pelleted, and the supernatant collected. aPC concentration was verified by Pierce BCA assay, and the aPC was either assayed directly, or frozen in aliquots at -80°C. All derivatives were analyzed by SDS-PAGE with either Coomassie-blue staining or Western Blot analysis to confirm complete activation (Laemmli, Nature 227:680-685, 1970).

Example 4Functional Characterization

The amidolytic activity of recombinant human protein C derivatives were determined by hydrolysis of the tri-peptide substrates S-2366 (Glu-Pro-Arg-p-nitroanilide), S-2238 (Pip-Pro-Arg-p-nitroanilide), and S-2288 (Ile-Pro-Arg-p-nitroanilide). The anti-coagulant activity is shown as measured clotting time in an aPTT at 500 ng mL⁻¹ aPC. Amidolytic activities were measured using the chromogenic substrate S-2366.

-36-

Assays were performed at 25°C, in Buffer A containing 1 mg mL⁻¹ BSA, 3 mM CaCl₂, and 0.5 nM aPC. Reactions (200 µL/well) were performed in a 96-well microtiter plate, and amidolytic activity was measured as the change in absorbance units/min at 405 nm as monitored in a ThermoMax kinetic micrometer plate reader. Kinetic constants were derived by fitting velocity data at varying substrate concentrations (16 µM to 2 mM) to the Michaelis-Menten equation. Changes in A₄₀₅ were converted to mmol product using a path length of 0.53 cm (Molecular Devices Technical Applications Bulletin 4-1), and an extinction coefficient for the released *p*-nitroanilide of 9620 M⁻¹ cm⁻¹ (Pfleiderer, Methods Enzymol 19:514-521, 1970). Anti-coagulant activity was assessed by measuring the prolongation of clotting time in the activated partial thromboplastin time clotting assay (Helena Laboratories). Clotting reactions were monitored in a ThermoMax kinetic microtiter plate reader, measuring the time to V_{max} in the change in turbidity.

-37-

We claim:

1. A human protein C derivative comprising SEQ ID NO: 1 wherein Asp at position 167 is substituted with Phe; Asp at position 172 is substituted with Lys and further comprising at least one amino acid substitution selected from the group consisting of:

His at position 10, Ser at position 11, or Ser at position 12 are independently substituted with any amino acid;

Gln at position 32 is substituted with Glu;

Asn at position 33 is substituted with Asp or Phe; and, amino acids at positions 194, 195, 228, 249, 254, 302, or 316 are substituted with an amino acid selected from Ser, Ala, Thr, His, Leu, Lys, Arg, Asn, Asp, Glu, Gly, and Gln.

2. The human protein C derivative of Claim 1, wherein said human protein C derivative is in its activated form.

3. The human protein C derivative of Claim 1 wherein Ser at position 11 is substituted with Gly; Gln at position 32 is substituted with Glu; Asn at position 33 is substituted with Asp; and, Leu at position 194 is replaced with Ser (SEQ ID NO: 3).

4. The human protein C derivative of Claim 1 wherein Ser at position 11 is substituted with Gly; Gln at position 32 is substituted with Glu; Asn at position 33 is substituted with Asp; and, Leu at position 194 is replaced with Ser; and Thr at position 254 is replaced with Ser (SEQ ID NO: 4).

-38-

5. The human protein C derivative of Claim 1 wherein Ser at position 11 is substituted with Gly; Gln at position 32 is substituted with Glu; and, Asn at position 33 is substituted with Asp (SEQ ID NO: 5).

6. The human protein C derivative of Claim 1 wherein His at position 10 is replaced with Gln; Ser at position 11 is replaced with Gly; Gln at position 32 is substituted with Glu; and, Asn at position 33 is substituted with Asp (SEQ ID NO: 6).

7. A recombinant DNA molecule encoding the human protein C derivative of any one of Claims 1 through 6.

8. A method of treating acute coronary syndromes and disease states predisposing to thrombosis which comprises: administering to a patient in need thereof a pharmaceutically effective amount of a human protein C derivative of Claim 1.

9. A method of treating vascular occlusive disorders and hypercoagulable states which comprises: administering to a patient in need thereof a pharmaceutically effective amount of a human protein C derivative of Claim 1.

10. A method of treating sepsis which comprises: administering to a patient in need thereof a pharmaceutically effective amount of a human protein C derivative of Claim 1 in combination with bactericidal permeability increasing protein or tissue factor pathway inhibitor.

-39-

11. A method of treating thrombotic disorders which comprises: administering to a patient in need thereof a pharmaceutically effective amount of an isolated human protein C derivative of Claim 1 in combination with an anti-platelet agent.

12. A method of treating protein C deficiency which comprises: administering to a patient in need thereof a pharmaceutically effective amount of a human protein C derivative of Claim 1.

13. A method of treating acute arterial thrombotic occlusion, thromboembolism, or stenosis in coronary, cerebral or peripheral arteries or in vascular grafts comprising: administering to a patient in need thereof a pharmaceutically effective amount of a human activated protein C derivative of Claim 1 in combination with a thrombolytic agent.

14. A method of treating human patients with genetically predisposed prothrombotic disorders comprising: administering gene therapy to said patients with a recombinant DNA molecule encoding a protein C derivative of Claim 1.

15. A method of treating thrombotic disorders comprising: administering a human activated protein C derivative of Claim 1 by local delivery through an intracoronary catheter.

-40-

16. The method of any one of Claims 8 through 17 wherein said human protein C derivative is selected from the group consisting of S11G:Q32E:N33D:D167F:D172K:L194S, S11G:Q32E:N33D:D167F:D172K:L194S:T254S, S11G:Q32E:N33D:D167F:D172K, or H10Q:S11G:Q32E:N33D:D167F:D172K.

17. A pharmaceutical composition comprising: a human protein C derivative of any one of Claims 1 through 6 in a pharmaceutically acceptable diluent.

18. The pharmaceutical composition of Claim 19 wherein said human protein C derivative is selected from the group consisting of S11G:Q32E:N33D:D167F:D172K:L194S, S11G:Q32E:N33D:D167F:D172K:L194S:T254S, S11G:Q32E:N33D:D167F:D172K, or H10Q:S11G:Q32E:N33D:D167F:D172K.

19. The use of the human activated protein C derivative of Claim 1 for the manufacture of a medicament for the treatment of acute coronary syndromes, vascular occlusive disorders and hypercoagulable states, sepsis in combination with bactericidal permeability increasing protein, thrombotic disorders, thrombotic disorders in combination with an anti-platelet agent, genetically predisposed prothrombotic disorders, and sepsis in combination with tissue factor pathway inhibitor.

20. A vector, comprising a nucleic acid according to Claim 7.

-41-

21. A host cell transformed by the vector according to Claim 20.

22. A method of producing a human protein C derivative of Claim 1 comprising:

- (a) transforming a host cell with a vector containing nucleic acid encoding a human protein C derivative;
- (b) culturing said host cell in a medium appropriate for expression of said human protein C derivative;
- (c) isolating said human protein C derivative from the culture medium; and,
- (d) activating said human protein C derivative.

23. The method according to Claim 22 wherein the nucleic acid encodes a human protein C derivative selected from the group consisting of

S11G:Q32E:N33D:D167F:D172K:L194S,
S11G:Q32E:N33D:D167F:D172K:L194S:T254S,
S11G:Q32E:N33D:D167F:D172K, or
H10Q:S11G:Q32E:N33D:D167F:D172K.

24. The method according to Claim 22 wherein said host cell is selected from the group consisting of 293 cells and AV12 cells.

SEQUENCE LISTING

<110> Gerlitz, Bruce E.
 Jones, Bryan E.
 Grinnell, Brian W

<120> PROTEIN C DERIVATIVES

<130> X-13611

<140>

<141>

<150> 60/181948

<151> 2000-02-11

<150> 60/189199

<151> 2000-03-14

<160> 12

<170> PatentIn Ver. 2.1

<210> 1

<211> 419

<212> PRT

<213> Hcmo sapiens

<400> 1

Ala	Asn	Ser	Phe	Leu	Glu	Glu	Leu	Arg	His	Ser	Ser	Leu	Glu	Arg	Glu	1	5	10	15
Cys	Ile	Glu	Glu	Ile	Cys	Asp	Phe	Glu	Glu	Ala	Lys	Glu	Ile	Phe	Gln	20	25	30	
Asn	Val	Asp	Asp	Thr	Leu	Ala	Phe	Trp	Ser	Lys	His	Val	Asp	Gly	Asp	35	40	45	
Gln	Cys	Leu	Val	Leu	Pro	Leu	Glu	His	Pro	Cys	Ala	Ser	Leu	Cys	Cys	50	55	60	
Gly	His	Gly	Thr	Cys	Ile	Asp	Gly	Ile	Gly	Ser	Phe	Ser	Cys	Asp	Cys	65	70	75	80
Arg	Ser	Gly	Trp	Glu	Gly	Arg	Phe	Cys	Gln	Arg	Glu	Val	Ser	Phe	Leu	85	90	95	
Asn	Cys	Ser	Leu	Asp	Asn	Gly	Gly	Cys	Thr	His	Tyr	Cys	Leu	Glu	Glu	100	105	110	
Val	Gly	Trp	Arg	Arg	Cys	Ser	Cys	Ala	Pro	Gly	Tyr	Lys	Leu	Gly	Asp	115	120	125	
Asp	Leu	Leu	Gln	Cys	His	Pro	Ala	Val	Lys	Phe	Pro	Cys	Gly	Arg	Pro	130	135	140	
Trp	Lys	Arg	Met	Glu	Lys	Lys	Arg	Ser	His	Leu	Lys	Arg	Asp	Thr	Glu	145	150	155	160
Asp	Gln	Glu	Asp	Gln	Val	Asp	Pro	Arg	Leu	Ile	Asp	Gly	Lys	Met	Thr	165	170	175	
Arg	Arg	Gly	Asp	Ser	Pro	Trp	Gln	Val	Val	Leu	Leu	Asp	Ser	Lys	Lys	180	185	190	
Lys	Leu	Ala	Cys	Gly	Ala	Val	Leu	Ile	His	Pro	Ser	Trp	Val	Leu	Thr				

195 200 205
 Ala Ala His Cys Met Asp Glu Ser Lys Lys Leu Leu Val Arg Leu Gly
 210 215 220
 Glu Tyr Asp Leu Arg Arg Trp Glu Lys Trp Glu Leu Asp Leu Asp Ile
 225 230 235 240
 Lys Glu Val Phe Val His Pro Asn Tyr Ser Lys Ser Thr Thr Asp Asn
 245 250 255
 Asp Ile Ala Leu Leu His Leu Ala Gln Pro Ala Thr Leu Ser Gln Thr
 260 265 270
 Ile Val Pro Ile Cys Leu Pro Asp Ser Gly Leu Ala Glu Arg Glu Leu
 275 280 285
 Asn Gln Ala Gly Gln Glu Thr Leu Val Thr Gly Trp Gly Tyr His Ser
 290 295 300
 Ser Arg Glu Lys Glu Ala Lys Arg Asn Arg Thr Phe Val Leu Asn Phe
 305 310 315 320
 Ile Lys Ile Pro Val Val Pro His Asn Glu Cys Ser Glu Val Met Ser
 325 330 335
 Asn Met Val Ser Glu Asn Met Leu Cys Ala Gly Ile Leu Gly Asp Arg
 340 345 350
 Gln Asp Ala Cys Glu Gly Asp Ser Gly Gly Pro Met Val Ala Ser Phe
 355 360 365
 His Gly Thr Trp Phe Leu Val Gly Leu Val Ser Trp Gly Glu Gly Cys
 370 375 380
 Gly Leu Leu His Asn Tyr Gly Val Tyr Thr Lys Val Ser Arg Tyr Leu
 385 390 395 400
 Asp Trp Ile His Gly His Ile Arg Asp Lys Glu Ala Pro Gln Lys Ser
 405 410 415
 Trp Ala Pro

<210> 2
 <211> 461
 <212> PRT
 <213> Homo sapiens

<400> 2
 Met Trp Gln Leu Thr Ser Leu Leu Leu Phe Val Ala Thr Trp Gly Ile
 1 5 10 15
 Ser Gly Thr Pro Ala Pro Leu Asp Ser Val Phe Ser Ser Ser Glu Arg
 20 25 30
 Ala His Gln Val Leu Arg Ile Arg Lys Arg Ala Asn Ser Phe Leu Glu
 35 40 45
 Glu Leu Arg His Ser Ser Leu Glu Arg Glu Cys Ile Glu Glu Ile Cys
 50 55 60
 Asp Phe Glu Glu Ala Lys Glu Ile Phe Gln Asn Val Asp Asp Thr Leu
 65 70 75 80
 Ala Phe Trp Ser Lys His Val Asp Gly Asp Gln Cys Leu Val Leu Pro

3

Ile Arg Asp Lys Glu Ala Pro Gln Lys Ser Trp Ala Pro
 450 455 460

<210> 3
 <211> 419
 <212> PRT
 <213> Homo sapiens

<400> 3
 Ala Asn Ser Phe Leu Glu Glu Leu Arg His Gly Ser Leu Glu Arg Glu
 1 5 10 15
 Cys Ile Glu Glu Ile Cys Asp Phe Glu Glu Ala Lys Glu Ile Phe Glu
 20 25 30
 Asp Val Asp Asp Thr Leu Ala Phe Trp Ser Lys His Val Asp Gly Asp
 35 40 45
 Gln Cys Leu Val Leu Pro Leu Glu His Pro Cys Ala Ser Leu Cys Cys
 50 55 60
 Gly His Gly Thr Cys Ile Asp Gly Ile Gly Ser Phe Ser Cys Asp Cys
 65 70 75 80
 Arg Ser Gly Trp Glu Gly Arg Phe Cys Gln Arg Glu Val Ser Phe Leu
 85 90 95
 Asn Cys Ser Leu Asp Asn Gly Gly Cys Thr His Tyr Cys Leu Glu Glu
 100 105 110
 Val Gly Trp Arg Arg Cys Ser Cys Ala Pro Gly Tyr Lys Leu Gly Asp
 115 120 125
 Asp Leu Leu Gln Cys His Pro Ala Val Lys Phe Pro Cys Gly Arg Pro
 130 135 140
 Trp Lys Arg Met Glu Lys Lys Arg Ser His Leu Lys Arg Asp Thr Glu
 145 150 155 160
 Asp Gln Glu Asp Gln Val Phe Pro Arg Leu Ile Lys Gly Lys Met Thr
 165 170 175
 Arg Arg Gly Asp Ser Pro Trp Gln Val Val Leu Leu Asp Ser Lys Lys
 180 185 190
 Lys Ser Ala Cys Gly Ala Val Leu Ile His Pro Ser Trp Val Leu Thr
 195 200 205
 Ala Ala His Cys Met Asp Glu Ser Lys Lys Leu Leu Val Arg Leu Gly
 210 215 220
 Glu Tyr Asp Leu Arg Arg Trp Glu Lys Trp Glu Leu Asp Leu Asp Ile
 225 230 235 240
 Lys Glu Val Phe Val His Pro Asn Tyr Ser Lys Ser Thr Thr Asp Asn
 245 250 255
 Asp Ile Ala Leu Leu His Leu Ala Gln Pro Ala Thr Leu Ser Gln Thr
 260 265 270
 Ile Val Pro Ile Cys Leu Pro Asp Ser Gly Leu Ala Glu Arg Glu Leu
 275 280 285
 Asn Gln Ala Gly Gln Glu Thr Leu Val Thr Gly Trp Gly Tyr His Ser
 290 295 300

Ser Arg Glu Lys Glu Ala Lys Arg Asn Arg Thr Phe Val Leu Asn Phe
 305 310 315 320
 Ile Lys Ile Pro Val Val Pro His Asn Glu Cys Ser Glu Val Met Ser
 325 330 335
 Asn Met Val Ser Glu Asn Met Leu Cys Ala Gly Ile Leu Gly Asp Arg
 340 345 350
 Gln Asp Ala Cys Glu Gly Asp Ser Gly Gly Pro Met Val Ala Ser Phe
 355 360 365
 His Gly Thr Trp Phe Leu Val Gly Leu Val Ser Trp Gly Glu Gly Cys
 370 375 380
 Gly Leu Leu His Asn Tyr Gly Val Tyr Thr Lys Val Ser Arg Tyr Leu
 385 390 395 400
 Asp Trp Ile His Gly His Ile Arg Asp Lys Glu Ala Pro Gln Lys Ser
 405 410 415
 Trp Ala Pro

<210> 4
 <211> 419
 <212> PRT
 <213> Homo sapiens

<400> 4
 Ala Asn Ser Phe Leu Glu Glu Leu Arg His Gly Ser Leu Glu Arg Glu
 1 5 10 15
 Cys Ile Glu Glu Ile Cys Asp Phe Glu Glu Ala Lys Glu Ile Phe Glu
 20 25 30
 Asp Val Asp Asp Thr Leu Ala Phe Trp Ser Lys His Val Asp Gly Asp
 35 40 45
 Gln Cys Leu Val Leu Pro Leu Glu His Pro Cys Ala Ser Leu Cys Cys
 50 55 60
 Gly His Gly Thr Cys Ile Asp Gly Ile Gly Ser Phe Ser Cys Asp Cys
 65 70 75 80
 Arg Ser Gly Trp Glu Gly Arg Phe Cys Gln Arg Glu Val Ser Phe Leu
 85 90 95
 Asn Cys Ser Leu Asp Asn Gly Gly Cys Thr His Tyr Cys Leu Glu Glu
 100 105 110
 Val Gly Trp Arg Arg Cys Ser Cys Ala Pro Gly Tyr Lys Leu Gly Asp
 115 120 125
 Asp Leu Leu Gln Cys His Pro Ala Val Lys Phe Pro Cys Gly Arg Pro
 130 135 140
 Trp Lys Arg Met Glu Lys Lys Arg Ser His Leu Lys Arg Asp Thr Glu
 145 150 155 160
 Asp Gln Glu Asp Gln Val Phe Pro Arg Leu Ile Lys Gly Lys Met Thr
 165 170 175
 Arg Arg Gly Asp Ser Pro Trp Gln Val Val Leu Leu Asp Ser Lys Lys
 180 185 190

Lys Ser Ala Cys Gly Ala Val Leu Ile His Pro Ser Trp Val Leu Thr
 195 200 205
 Ala Ala His Cys Met Asp Glu Ser Lys Lys Leu Leu Val Arg Leu Gly
 210 215 220
 Glu Tyr Asp Leu Arg Arg Trp Glu Lys Trp Glu Leu Asp Leu Asp Ile
 225 230 235 240
 Lys Glu Val Phe Val His Pro Asn Tyr Ser Lys Ser Thr Ser Asp Asn
 245 250 255
 Asp Ile Ala Leu Leu His Leu Ala Gln Pro Ala Thr Leu Ser Gln Thr
 260 265 270
 Ile Val Pro Ile Cys Leu Pro Asp Ser Gly Leu Ala Glu Arg Glu Leu
 275 280 285
 Asn Gln Ala Gly Gln Glu Thr Leu Val Thr Gly Trp Gly Tyr His Ser
 290 295 300
 Ser Arg Glu Lys Glu Ala Lys Arg Asn Arg Thr Phe Val Leu Asn Phe
 305 310 315 320
 Ile Lys Ile Pro Val Val Pro His Asn Glu Cys Ser Glu Val Met Ser
 325 330 335
 Asn Met Val Ser Glu Asn Met Leu Cys Ala Gly Ile Leu Gly Asp Arg
 340 345 350
 Gln Asp Ala Cys Glu Gly Asp Ser Gly Gly Pro Met Val Ala Ser Phe
 355 360 365
 His Gly Thr Trp Phe Leu Val Gly Leu Val Ser Trp Gly Glu Gly Cys
 370 375 380
 Gly Leu Leu His Asn Tyr Gly Val Tyr Thr Lys Val Ser Arg Tyr Leu
 385 390 395 400
 Asp Trp Ile His Gly His Ile Arg Asp Lys Glu Ala Pro Gln Lys Ser
 405 410 415
 Trp Ala Pro

<210> 5
 <211> 419
 <212> PRT
 <213> Homo sapiens

<400> 5
 Ala Asn Ser Phe Leu Glu Glu Leu Arg His Gly Ser Leu Glu Arg Glu
 1 5 10 15
 Cys Ile Glu Glu Ile Cys Asp Phe Glu Glu Ala Lys Glu Ile Phe Glu
 20 25 30
 Asp Val Asp Asp Thr Leu Ala Phe Trp Ser Lys His Val Asp Gly Asp
 35 40 45
 Gln Cys Leu Val Leu Pro Leu Glu His Pro Cys Ala Ser Leu Cys Cys
 50 55 60
 Gly His Gly Thr Cys Ile Asp Gly Ile Gly Ser Phe Ser Cys Asp Cys
 65 70 75 80

Arg Ser Gly Trp Glu Gly Arg Phe Cys Gln Arg Glu Val Ser Phe Leu
 85 90 95
 Asn Cys Ser Leu Asp Asn Gly Gly Cys Thr His Tyr Cys Leu Glu Glu
 100 105 110
 Val Gly Trp Arg Arg Cys Ser Cys Ala Pro Gly Tyr Lys Leu Gly Asp
 115 120 125
 Asp Leu Leu Gln Cys His Pro Ala Val Lys Phe Pro Cys Gly Arg Pro
 130 135 140
 Trp Lys Arg Met Glu Lys Lys Arg Ser His Leu Lys Arg Asp Thr Glu
 145 150 155 160
 Asp Gln Glu Asp Gln Val Phe Pro Arg Leu Ile Lys Gly Lys Met Thr
 165 170 175
 Arg Arg Gly Asp Ser Pro Trp Gln Val Val Leu Leu Asp Ser Lys Lys
 180 185 190
 Lys Leu Ala Cys Gly Ala Val Leu Ile His Pro Ser Trp Val Leu Thr
 195 200 205
 Ala Ala His Cys Met Asp Glu Ser Lys Lys Leu Leu Val Arg Leu Gly
 210 215 220
 Glu Tyr Asp Leu Arg Arg Trp Glu Lys Trp Glu Leu Asp Leu Asp Ile
 225 230 235 240
 Lys Glu Val Phe Val His Pro Asn Tyr Ser Lys Ser Thr Thr Asp Asn
 245 250 255
 Asp Ile Ala Leu Leu His Leu Ala Gln Pro Ala Thr Leu Ser Gln Thr
 260 265 270
 Ile Val Pro Ile Cys Leu Pro Asp Ser Gly Leu Ala Glu Arg Glu Leu
 275 280 285
 Asn Gln Ala Gly Gln Glu Thr Leu Val Thr Gly Trp Gly Tyr His Ser
 290 295 300
 Ser Arg Glu Lys Glu Ala Lys Arg Asn Arg Thr Phe Val Leu Asn Phe
 305 310 315 320
 Ile Lys Ile Pro Val Val Pro His Asn Glu Cys Ser Glu Val Met Ser
 325 330 335
 Asn Met Val Ser Glu Asn Met Leu Cys Ala Gly Ile Leu Gly Asp Arg
 340 345 350
 Gln Asp Ala Cys Glu Gly Asp Ser Gly Gly Pro Met Val Ala Ser Phe
 355 360 365
 His Gly Thr Trp Phe Leu Val Gly Leu Val Ser Trp Gly Glu Gly Cys
 370 375 380
 Gly Leu Leu His Asn Tyr Gly Val Tyr Thr Lys Val Ser Arg Tyr Leu
 385 390 395 400
 Asp Trp Ile His Gly His Ile Arg Asp Lys Glu Ala Pro Gln Lys Ser
 405 410 415
 Trp Ala Pro

<210> 6
 <211> 419
 <212> PRT
 <213> Homo sapiens

<400> 6
 Ala Asn Ser Phe Leu Glu Glu Leu Arg Gln Gly Ser Leu Glu Arg Glu
 1 5 10 15
 Cys Ile Glu Glu Ile Cys Asp Phe Glu Glu Ala Lys Glu Ile Phe Glu
 20 25 30
 Asp Val Asp Asp Thr Leu Ala Phe Trp Ser Lys His Val Asp Gly Asp
 35 40 45
 Gln Cys Leu Val Leu Pro Leu Glu His Pro Cys Ala Ser Leu Cys Cys
 50 55 60
 Gly His Gly Thr Cys Ile Asp Gly Ile Gly Ser Phe Ser Cys Asp Cys
 65 70 75 80
 Arg Ser Gly Trp Glu Gly Arg Phe Cys Gln Arg Glu Val Ser Phe Leu
 85 90 95
 Asn Cys Ser Leu Asp Asn Gly Gly Cys Thr His Tyr Cys Leu Glu Glu
 100 105 110
 Val Gly Trp Arg Arg Cys Ser Cys Ala Pro Gly Tyr Lys Leu Gly Asp
 115 120 125
 Asp Leu Leu Gln Cys His Pro Ala Val Lys Phe Pro Cys Gly Arg Pro
 130 135 140
 Trp Lys Arg Met Glu Lys Lys Arg Ser His Leu Lys Arg Asp Thr Glu
 145 150 155 160
 Asp Gln Glu Asp Gln Val Phe Pro Arg Leu Ile Lys Gly Lys Met Thr
 165 170 175
 Arg Arg Gly Asp Ser Pro Trp Gln Val Val Leu Leu Asp Ser Lys Lys
 180 185 190
 Lys Leu Ala Cys Gly Ala Val Leu Ile His Pro Ser Trp Val Leu Thr
 195 200 205
 Ala Ala His Cys Met Asp Glu Ser Lys Lys Leu Leu Val Arg Leu Gly
 210 215 220
 Glu Tyr Asp Leu Arg Arg Trp Glu Lys Trp Glu Leu Asp Leu Asp Ile
 225 230 235 240
 Lys Glu Val Phe Val His Pro Asn Tyr Ser Lys Ser Thr Thr Asp Asn
 245 250 255
 Asp Ile Ala Leu Leu His Leu Ala Gln Pro Ala Thr Leu Ser Gln Thr
 260 265 270
 Ile Val Pro Ile Cys Leu Pro Asp Ser Gly Leu Ala Glu Arg Glu Leu
 275 280 285
 Asn Gln Ala Gly Gln Glu Thr Leu Val Thr Gly Trp Gly Tyr His Ser
 290 295 300
 Ser Arg Glu Lys Glu Ala Lys Arg Asn Arg Thr Phe Val Leu Asn Phe
 305 310 315 320

```
<210> 7
<211> 1260
<212> DNA
<213> Homo sapiens
```

<400> 7						
gccaaactcct	tctctggagga	gctccgtcac	agcagccttg	agcgggagtg	catagaggag	60
atctgtgact	tcgaggaggc	caaggaaatt	ttccaaaatg	tggatgacac	actggccttc	120
tgggtccaagc	acgtcgacgg	tgaccagtgc	ttgggtcttg	ccttgaggca	cccgtgcgcc	180
agcctgtgct	gcgggcacgg	cacgtgcata	gacggcatcg	gcagcttcag	ctgcgactgc	240
cgcagcgggt	gggaggggcg	cttctgccag	cgcgagggtga	gcttcctcaa	ttgctcgctg	300
gacaacggcg	gctgcacgca	ttactgccta	gaggagggtg	gctggcggcg	ctgtagctgt	360
gcgcctgggt	acaagctggg	ggacgacctc	ctgcagtgtc	accccgagtg	gaagtccctt	420
tgtgggaggc	cctggaagcg	gatggagaag	aagcgcagtc	acctgaaacg	agacacagaa	480
gaccaagaag	accaagtaga	tccgcggctc	attgatggga	agatgaccag	gcggggagac	540
agcccctggc	aggtggtcct	gctggactca	aagaagaagc	tggcctgcgg	ggcagtgctc	600
atccacccct	cctgggtgct	gacagcggcc	cactgcatgg	atgagtccaa	gaagctcctt	660
gtcaggcttg	gagagtatga	cctgcggcgc	tgggagaagt	gggagctgga	cctggacatc	720
aaggagggtct	tcgtccaccc	caactacagc	aagagcacca	ccgacaatga	catcgcactg	780
ctgcacctgg	cccagccgcg	cacctctctg	cagaccatag	tgccatcttg	cctcccgga	840
agcggccttg	cagagcgcga	gctcaatcag	gccggccagg	agaccctcgt	gacgggctgg	900
ggctaccaca	gcagccgaga	gaaggaggcc	aagagaaacc	gcaccttcgt	cctcaacttc	960
atcaagattc	ccgtgggtccc	gcacaatgag	tgcagcgagg	tcattgagcaa	catggtgtct	1020
gagaacatgc	tgtgtgcggg	cattctcggg	gaccggcagg	atgcctgcga	gggcgacagt	1080
ggggggccca	tggtcgcctc	cttcacggc	acctgggttc	tgggtgggct	ggtgagctgg	1140
ggtgagggct	gtggggtcct	tcacaactac	ggcggtttaca	ccaaagtcag	ccgtacctc	1200

gactggatcc atgggcacat cagagacaag gaagcccccc agaagagctg ggcaccttag 1260

<210> 8
<211> 1386
<212> DNA
<213> Homo sapiens

<400> 8
atgtggcagc tcacaagcct cctgctgttc gtggccacct ggggaatttc cggcacacca 60
gtcctctctg actcagtgtt ctccagcagc gagcgtgccc accaggtgct gcggatccgc 120
aaacgtgcc aactccttcct ggaggagctc cgtcacagca gcctggagcg ggagtgcata 180
gaggagatct gtgacttcga ggaggccaag gaaattttcc aaaatgtgga tgacacactg 240
gccttctggt ccaagcacgt cgacggtgac cagtgtcttg tcttgccctt ggagcaccgc 300
tgcccgagcc tgtgctgcgg gcacggcacg tgcacgacg gcacggcag cttcagctgc 360
gactgccgca gcggctggga gggccgcttc tgccagcgcg aggtgagctt cctcaattgc 420
tcgctggaca acggcggtct caccgattac tgcctagagg aggtgggctg gcggcgctgt 480
agctgtgcgc ctggctacaa gctgggggac gacctcctgc agtgtcacc cgcagtgaag 540
ttcccttggt ggaggccctg gaagcggatg gagaagaagc gcagtcacct gaaacgagac 600
acagaagacc aagaagacca agtagatccg cggctcattg atgggaagat gaccaggcgg 660
ggagacagcc cctggcaggt ggtcctgctg gactcaaaga agaagctggc ctgcggggca 720
gtgtcatcc accctcctg ggtgctgaca gcggccact gcatggatga gtccaagaag 780
ctccttgta ggcttgaga gtatgacctg cggcgctggg agaagtggga gctggacctg 840
gacatcaagg aggtcttcgt ccacccaac tacagcaaga gcaccaccga caatgacatc 900
gcactgctgc acctggcca gcccgccacc ctctcgaga ccatagtgc catctgcctc 960
ccggacagcg gccttgaga gcgcgagctc aatcaggccg gccaggagac cctcgtgacg 1020
ggctggggct accacagcag ccgagagaag gaggccaaga gaaaccgcac cttcgtcctc 1080
aacttcatca agattcccgt ggtccgcac aatgagtga gcgaggtcat gagcaacatg 1140
gtgtctgaga acatgctgtg tgcgggcac ctccgggacc ggcaggatgc ctgcgagggc 1200
gacagtggg ggcccatggt cgcctcctc caccggacct ggttcctggt gggcctggtg 1260
agctgggggt agggctgtgg gtccttcac aactacggcg ttacaccaa agtcagccgc 1320
tacctcgact ggatccatgg gcacatcaga gacaaggaag cccccagaa gagctgggca 1380
ccttag 1386

<210> 9
<211> 1386
<212> DNA
<213> Homo sapiens

<400> 9

```

atgtggcagc tcacaagcct cctgctgttc gtggccacct ggggaatttc cggcacacca 60
gctcctcttg actcagtgtt ctccagcagc gagcgtgccc accaggtgct gcggatccgc 120
aaacgtgcca actccttcct ggaggagctc cgtcacggga gcctggagcg ggagtgcata 180
gaggagatct gtgacttcga ggaggccaag gaaattttcg aagatgtgga tgacacactg 240
gccttctggt ccaagcacgt cgacggtgac cagtgccttg tcttgccctt ggagcaccgc 300
tgcgccagcc tgtgctgcgg gcacggcacg tgcacgacg gcatcggcag cttcagctgc 360
gactgccgca gcggctggga gggccgcttc tgccagcgcg aggtgagctt cctcaattgc 420
tctctggaca acggcggtg cacgcattac tgcctagagg aggtgggctg gcggcgctgt 480
agctgtgctc ctggctacaa gctgggggac gacctcctgc agtgtcacc cgcagtgaag 540
ttcccttctg ggaggccctg gaagcggatg gagaagaagc gcagtcacct gaaacgagac 600
acagaagacc aagaagacca agtattcccg cggctcatta aggggaagat gaccaggcgg 660
ggagacagcc cctggcaggt ggtcctgctg gactcaaaga agaagtcgcg ctgcggggca 720
gtgctcatcc accctcctg ggtgctgaca gcggccact gcatggatga gtccaagaag 780
ctccttgctc ggcttgaga gtatgacctg cggcgctggg agaagtgga gctggacctg 840
gacatcaagg aggtcttcgt ccacccaac tacagcaaga gcaccaccga caatgacatc 900
gcactgctgc acctggccca gcccgccacc ctctgcaga ccatagtgc catctgcctc 960
ccggacagcg gccttgaga gcgcgagctc aatcaggccg gccaggagac cctcgtgacg 1020
ggctggggct accacagcag ccgagagaag gaggccaaga gaaaccgcac cttcgtcctc 1080
aacttcacat agattccctg ggtcccgac aatgagtgc gcgaggatc gagcaacatg 1140
gtgtctgaga acatgctgtg tgcgggcatc ctccgggacc ggcaggatgc ctgcgagggc 1200
gacagtgggg ggccatggt cgcctccttc cacggcacct ggttcctggt gggcctgggtg 1260
agctgggggt agggctgtgg gctccttcac aactacggcg ttacaccaa agtcagccgc 1320
tacctcgact ggatccatgg gcacatcaga gacaaggaag cccccagaa gagctgggca 1380
ccttag 1386

```

<210> 10
 <211> 1386
 <212> DNA
 <213> Homo sapiens

```

<400> 10
atgtggcagc tcacaagcct cctgctgttc gtggccacct ggggaatttc cggcacacca 60
gctcctcttg actcagtgtt ctccagcagc gagcgtgccc accaggtgct gcggatccgc 120
aaacgtgcca actccttcct ggaggagctc cgtcacggga gcctggagcg ggagtgcata 180
gaggagatct gtgacttcga ggaggccaag gaaattttcg aagatgtgga tgacacactg 240
gccttctggt ccaagcacgt cgacggtgac cagtgccttg tcttgccctt ggagcaccgc 300
tgcgccagcc tgtgctgcgg gcacggcacg tgcacgacg gcatcggcag cttcagctgc 360

```

gactgccgca gcggctggga gggccgcttc tgccagcgcg aggtgagctt cctcaattgc 420
 tctctggaca acggcggtg cagcattac tgccatagagg aggtgggctg gcggcgctgt 480
 agctgtgctg ctggctacaa gctgggggac gacctcctgc agtgtcaccg cgagtgaaag 540
 ttcccttgtg ggaggccctg gaagcggatg gagaagaagc gcagtcacct gaaacgagac 600
 acagaagacc aagaagacca agtattcccg cggctcatta aggggaagat gaccaggcgg 660
 ggagacagcc cctggcaggt ggtcctgctg gactcaaaga agaagtccgc ctgcggggca 720
 gtgctcatcc acccctcctg ggtgctgaca gcggccact gcattgatga gtccaagaag 780
 ctccctgtca ggcttggaga gtatgacctg cggcgctggg agaagtggga gctggacctg 840
 gacatcaagg aggtcttctg ccaccccaac tacagcaaga gcaccagcga caatgacatc 900
 gcaactgctg acctggccca gcccgccacc ctctcgaga ccatagtgc catctgcctc 960
 ccggacagcg gccttgcaga gcgcgagctc aatcaggcgc gccaggagac cctcgtgacg 1020
 ggctggggct accacagcag ccgagagaag gaggccaaga gaaaccgcac ctctgtcctc 1080
 aacttcatca agattcccg ggtcccgac aatgagtga gcgaggtcat gagcaacatg 1140
 gtgtctgaga acatgctgtg tcggggcacc ctccgggacc ggcaggatgc ctgcgagggc 1200
 gacagtgggg ggcccatggt cgctccttc caccggcact ggttcctggt gggcctggtg 1260
 agctgggggt agggctgtgg gctccttcac aactacggcg ttacaccaa agtcagccgc 1320
 tacctcgact ggatccatgg gcacatcaga gacaaggaag ccccccagaa gagctgggca 1380
 ccttag 1386

<210> 11
 <211> 1386
 <212> DNA
 <213> Homo sapiens

<400> 11
 atgtggcagc tcacaagcct cctgctgttc gtggccacct ggggaatttc cggcacacca 60
 gctcctcttg actcagtgtt ctccagcagc gagcgtgccc accagggtgct gcggatccgc 120
 aaacgtgcca actccttcct ggaggagctc cgtcaaggga gcctggagcg ggagtgcata 180
 gaggagatct gtgacttga ggaggccaag gaaattttcg aagatgtgga tgacacactg 240
 gccttctggt ccaagcacgt cgacggtgac cagtgtctgg tcttgccctt ggagcaccgc 300
 tgccgagcc tgtgctgcgg gcacggcacg tgcatcgacg gcacggcag cttcagctgc 360
 gactgccgca gcggctggga gggccgcttc tgccagcgcg aggtgagctt cctcaattgc 420
 tctctggaca acggcggtg cagcattac tgccatagagg aggtgggctg gcggcgctgt 480
 agctgtgctg ctggctacaa gctgggggac gacctcctgc agtgtcaccg cgagtgaaag 540
 ttcccttgtg ggaggccctg gaagcggatg gagaagaagc gcagtcacct gaaacgagac 600
 acagaagacc aagaagacca agtattcccg cggctcatta aggggaagat gaccaggcgg 660

ggagacagcc cctggcaggt ggtcctgctg gactcaaaga agaagctggc ctgcggggca 720
 gtgtcatcc acccctcctg ggtgctgaca gcgcccact gcatggatga gtccaagaag 780
 ctccttgta ggcttgaga gtatgacctg cggcgctggg agaagtggga gctggacctg 840
 gacatcaagg aggtcttcgt ccacccaac tacagcaaga gcaccaccga caatgacatc 900
 gcactgctgc acctggccca gcccgccacc ctctcgaga ccatagtgcc catctgcctc 960
 ccggacagcg gccttgaga gcgcgagctc aatcaggccg gccaggagac cctcgtgacg 1020
 ggctggggct accacagcag ccgagagaag gaggccaaga gaaaccgcac ctctgcctc 1080
 aacttcatca agattcccgt ggtcccgac aatgagtga gcgaggtcat gagcaacatg 1140
 gtgtctgaga acatgctgtg tgcgggcac ctccgggacc ggcaggatgc ctgcgagggc 1200
 gacagtgggg ggcccatggt cgcctcctc caccgcacct ggttcctggt gggcctgggtg 1260
 agctgggggtg agggctgtgg gctccttcac aactacggcg ttacaccaa agtcagccgc 1320
 tacctcgact ggatccatgg gcacatcaga gacaaggaag ccccccagaa gagctgggca 1380
 ccttag 1386

<210> 12

<211> 1386

<212> DNA

<213> Homo sapiens

<400> 12

atgtggcagc tcacaagcct cctgctgttc gtggccacct ggggaatttc cggcacacca 60
 gctcctcttg actcagtgtt ctccagcagc gagcgtgccc accaggtgct gcggatccgc 120
 aaacgtgcca actccttcct ggaggagctc cgtcaagga gcctggagcg ggagtgcata 180
 gaggagatct gtgacttoga ggaggccaag gaaattttcg aagatgtgga tgacacactg 240
 gccttctggt ccaagcacgt cgacggtgac cagtgccttg tcttgccctt ggagcaccgc 300
 tgcgccagcc tgtgctgcgg gcacggcacg tgcacgacg gcacggcag cttcagctgc 360
 gactgccgca gcggctggga gggccgcttc tgccagcgcg aggtgagctt cctcaattgc 420
 tctctggaca acggcggtg caccgattac tgcctagagg aggtgggctg gcggcgctgt 480
 agctgtgcgc ctggctacaa gctgggggac gacctcctgc agtgtcacc cgagtgaaag 540
 ttcccttggt ggaggccctg gaagcggatg gagaagaagc gcagtcacct gaaacgagac 600
 acagaagacc aagaagacca agtattcccg cggctcatta aggggaagat gaccaggcgg 660
 ggagacagcc cctggcaggt ggtcctgctg gactcaaaga agaagctggc ctgcggggca 720
 gtgtcatcc acccctcctg ggtgctgaca gcgcccact gcatggatga gtccaagaag 780
 ctccttgta ggcttgaga gtatgacctg cggcgctggg agaagtggga gctggacctg 840
 gacatcaagg aggtcttcgt ccacccaac tacagcaaga gcaccaccga caatgacatc 900
 gcactgctgc acctggccca gcccgccacc ctctcgaga ccatagtgcc catctgcctc 960
 ccggacagcg gccttgaga gcgcgagctc aatcaggccg gccaggagac cctcgtgacg 1020

ggctggggct accacagcag ccgagagaag gagggccaaga gaaaccgcac cttcgtcctc 1080
aacttcatca agattcccggt ggtcccgcac aatgagtgca gcgaggatcat gagcaacatg 1140
gtgtctgaga acatgctgtg tgcgggcatc ctcggggacc ggcaggatgc ctgcgagggc 1200
gacagtgggg ggcccatggt cgcctccttc cacggcacct ggttcctggt gggcctggtg 1260
agctgggggtg agggctgtgg gtccttcac aactacggcg ttacaccaa agtcagccgc 1320
tacctcgact ggatccatgg gcacatcaga gacaaggaag cccccagaa gagctgggca 1380
ccttag 1386

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/01221

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C12N9/64 C12N15/85 A61K38/48		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12N A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 358 932 A (FOSTER DONALD C ET AL) 25 October 1994 (1994-10-25) cited in the application see whole doc. esp. claims ---	1-24
X	US 5 453 373 A (GERLITZ BRUCE E ET AL) 26 September 1995 (1995-09-26) cited in the application see whole doc. esp. col.1, 1.56-col.3, 1.35 ---	1-24
A	EP 0 443 874 A (LILLY CO ELI) 28 August 1991 (1991-08-28) the whole document --- -/--	
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
Special categories of cited documents: *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *G* document member of the same patent family		
Date of the actual completion of the international search 27 June 2001		Date of mailing of the international search report 09/07/2001
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel: (+31-70) 340-2040, Tx 31 651 epo nl. Fax: (+31-70) 340-3016		Authorized officer Mueller, F

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/01221

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>SHEN LEI ET AL: "Enhancement of human protein C function by site-directed mutagenesis of the gamma-carboxyglutamic acid domain"</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US,</p> <p>vol. 273, no. 47,</p> <p>20 November 1998 (1998-11-20), pages 31086-31091, XP002149742</p> <p>ISSN: 0021-9258</p> <p>cited in the application</p> <p>the whole document</p> <p>-----</p>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 01/01221

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5358932 A	25-10-1994	US 5753224 A AT 163048 T AU 7168591 A CA 2071630 A DE 69032029 D DE 69032029 T DK 506821 T EP 0506821 A ES 2113878 T GR 3026414 T JP 6502986 T WO 9109960 A US 5766921 A	19-05-1998 15-02-1998 24-07-1991 30-06-1991 12-03-1998 20-08-1998 30-03-1998 07-10-1992 16-05-1998 30-06-1998 07-04-1994 11-07-1991 16-06-1998
US 5453373 A	26-09-1995	AU 3869993 A BR 9301944 A CA 2096604 A CN 1080658 A CZ 9300948 A EP 0575054 A FI 932282 A HU 69615 A,B JP 6080698 A MX 9302917 A NO 931840 A NZ 247651 A PH 29911 A RU 2122004 C	25-11-1993 30-11-1993 22-11-1993 12-01-1994 19-01-1994 22-12-1993 22-11-1993 28-09-1995 22-03-1994 01-11-1993 22-11-1993 28-03-1995 16-09-1996 20-11-1998
EP 0443874 A	28-08-1991	AT 153069 T AU 645776 B AU 7130791 A CA 2036894 A CN 1055560 A DE 69126049 D DE 69126049 T DK 443874 T ES 2101716 T FI 910854 A GR 3023756 T HU 56877 A IE 910611 A JP 3004375 B JP 4211380 A MX 24651 A NO 910719 A NZ 237199 A PT 96853 A SU 1838411 A US 5460953 A ZA 9101328 A	15-05-1997 27-01-1994 29-08-1991 24-08-1991 23-10-1991 19-06-1997 25-09-1997 17-11-1997 16-07-1997 24-08-1991 30-09-1997 28-10-1991 28-08-1991 31-01-2000 03-08-1992 28-02-1994 26-08-1991 28-04-1993 31-10-1991 30-08-1993 24-10-1995 28-10-1992

22.6607

THIS PAGE BLANK (USPTO)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)